REMARKS

Claims 1-7, 9, 11, 13-24, 26-30, and 33-43 were pending in the subject application. Applicants have hereinabove cancelled claims 1-26, 29-32, 34-36, 38-39, and 41-43 without prejudice or disclaimer to their right to pursue the subject matter of these claims in this or a future application. In addition, applicants have hereinabove amended claim 40 and added new claims 44-52. Support for these amendments may be found inter alia in the specification as follows: claim 44: cancelled claims 15 and 16; claim 45: cancelled claim 17; claim 46: cancelled claim 6; claim 47: cancelled claim 5; claim 48: cancelled claim 7; claim 49: cancelled claim 11; claim 50: cancelled claims 1 and 2; claim 51: cancelled claim 3; and claim 52: cancelled claim 4. The remaining changes to the claims merely introduce minor grammatical and format changes. In making these amendments, applicants neither concede the correctness of the Examiner's rejections nor abandon their right to pursue embodiments of the instant invention no longer claimed in this application. These amendments do not involve any issue of new matter. Therefore, entry of this Amendment is respectfully requested such that claims 27, 28, 33, 37, 40, and 44-52 will be pending and under examination.

In view of the preceding amendments and the arguments set forth below, applicants maintain that the Examiner's grounds of rejection have been overcome and respectfully request that the Examiner reconsider and withdraw these rejections.

Formalities

Applicants acknowledge the Examiner's withdrawal of the objection to claims 2, 5, 16, 21, and 29.

Applicants also acknowledge the Examiner's withdrawal of the rejection of claims 1-7, 9, 11, 13-19, 21, 25, and 27-30 under 35 U.S.C. §112, 2nd paragraph.

Election/Restrictions

The Examiner stated that applicants continue to traverse the withdrawal of non-elected claims 8, 10, 12, 31, and 32 in the paper filed September 17, 2004. The Examiner stated that the restriction requirement of Groups II-VIII and X, claims 8, 10, 12, 31, 32, is still deemed proper and remains final. The Examiner stated that new claims 35, 36, and 39 are also withdrawn from consideration as they are drawn to non-elected Group II.

In response, applicants respectfully traverse the Examiner's restriction. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 8, 10, 12, 31, 32, 35, 36, and 39 without prejudice or disclaimer to their right to pursue these claims in the future.

Claim Objections

The Examiner maintained the objection to claims 17-19 for the reasons of record stated in the Office action mailed March 18, 2004. The Examiner stated that applicants' arguments have been

fully considered but are not found fully persuasive.

In response, applicants respectfully traverse the Examiner's rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 17-19 without prejudice or disclaimer to their right to pursue these claims in the future. Thus, this ground of rejection is now moot.

Claim Rejections Under 35 U.S.C. §112, Second Paragraph

The Examiner maintained the rejection of claims 20, 22-24, 26, and 40-42 under 35 U.S.C. §112, second paragraph, as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention for the reasons of record stated in the office action mailed March 18, 2004.

In response, applicants respectfully traverse the Examiner's rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 20, 22-24, 26, 41 and 42 without prejudice or disclaimer to their right to pursue these claims in the future. Thus, this ground of rejection is now moot.

In response to the Examiner's rejection of claim 40 on this ground, applicants, without conceding the correctness of the Examiner's rejection but to expedite prosecution of the subject application, have hereinabove amended claim 40 such that it no longer recites "or" after step (b).

In view of the above remarks, applicants maintain that claim 40 as amended satisfies the requirements of 35 U.S.C. §112, second paragraph.

Claim Rejections Under 35 U.S.C. §112, First Paragraph

The Examiner rejected claims 20, 22-24, and 26 under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner stated that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention, for the reasons of record stated in the office action mailed March 18,2004. The Examiner stated that applicants' arguments were fully considered but were not found fully persuasive.

The Examiner also rejected claims 6, 11, 20, 22-24, 26, 30, 34, 38 and 41 under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for an expression silencing system comprising a nucleotide sequence encoding the T7 RNA polymerase, and pT7, allegedly does not reasonably provide enablement for functional equivalents or fragments of nucleotide sequences encoding T7 RNA polymerase, pT7, and the NOS terminator; the expression silencing system wherein the target sequence is a non-coding sequence in the plant genome. The Examiner stated that the specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims, for the reasons of record stated in the office action mailed March 18, 2004. The Examiner stated that applicants' arguments were fully considered but were not found

fully persuasive.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 6, 11, 20, 22-24, 26, 30, 34, 38 and 41 without prejudice or disclaimer to their right to pursue these claims in the future. Thus, this ground of rejection is now moot.

Claim Rejections Under 35 U.S.C. §102(b)

The Examiner maintained the rejection of claims 1-7, 13-15, 17 and 43 under 35 U.S.C. §102(b) as being anticipated by Lassner et al., for the reasons of record stated in the office action mailed March 18, 2004. The Examiner stated that applicants' arguments are fully considered but are not found fully persuasive.

In response, applicants respectfully traverse the Examiner's above rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 1-7, 13-15, 17 and 43 without prejudice or disclaimer to their right to pursue these claims in the future. Thus, this ground of rejection is now moot.

Claim Rejections Under 35 U.S.C. §103(a)

The Examiner rejected claims 1-7, 9, 13-17, 20-24, 26, 27-30, 33, 34, 37, 38, and 40-43 under 35 U.S.C. §103(a) as allegedly being unpatentable over Lassner et al. in combination with Blockland et al., (*Plant J.*, 1994, Vol. 6, pages 861-877), and Palauqui et al.

(*EMBO J.*, 1997, vol. 16, pages 4738-4745), for the reasons of record stated in the office action mailed March 18, 2004. The Examiner stated that applicants' arguments are fully considered but are not found fully persuasive.

The Examiner stated that applicants argued that a *prima facie* case of obviousness was not made out, and submitted a declaration under 37 C.F.R. §1.132, signed by co-inventor Dr. Ilan Sela, in support. The Examiner stated that the declaration states that the T7 silencing system differs from other silencing systems in that no siRNA could be detected, the silencing signal was not transduced across grafts, and the viral silencing suppressor He-Pro could not overcome the silencing effect. The Examiner stated that the declaration states that the T7 silencing system activity was confined to the nucleus, as silenced genes were methylated and pertinent siRNAs were detected in nuclear extracts, and dicer activity was enhanced (response, pages 50-51; declaration, item 21). The Examiner stated that however, these differences are not described in the specification as filed, nor are they encompassed by the claims. The Examiner stated that further, the information in the declaration regarding the inability of the silencing signal to transduce across grafts (also discussed in items 27-30) directly contradicts the instant specification. The Examiner stated that Example 4 of the specification teaches that in 3 of 6 plants tested, the silencing signal did transduce across grafts. The Examiner stated that page 4 of the specification states that "the invention relates to a method for producing a transgenic plant carrying a substantially silenced target sequence by grafting a plant, or parts thereof, carrying and expressing said silent target sequence on a transgenic plant obtained by the process of the invention." The Examiner stated that further, it is noted original claim 25 required grafting, and this claim was

only cancelled in applicants' most recent response.

The Examiner stated that the declaration also points out supposed deficiencies of Blockland et al. (items 22-25). The Examiner stated that however, applicants are arguing against the reference alone, not in combination with the other reference. The Examiner stated that one cannot show nonobviousness by attacking references individually where the rejections are based on combinations of references. The Examiner stated that the declaration also states that another unique characteristic of the T7 silencing system is the capacity of using only a short homology fragment, around 70 nucleotides (item 26). The Examiner stated that however, the claims are not limited to using only such fragments.

In response, applicants respectfully traverse the Examiner's rejection. Nevertheless, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have hereinabove cancelled claims 1-7, 9, 13-17, 20-24, 26, 29-30, 34, 38, and 41-43 without prejudice or disclaimer to their right to pursue these claims in the future. Thus, this ground of rejection is now moot.

In response to the Examiner's rejection of claims 27, 28, 33, 37 and 40, applicants note that the now claimed invention is directed to methods of silencing the expression of a target gene in a plant. In this regard, applicants respectfully traverse the Examiner's rejection, and again maintain that the Examiner has failed to establish a prima facie case of obviousness against the rejected claims.

To establish a prima facie case of obviousness, the Examiner must

demonstrate three things with respect to each claim. First, the cited references, when combined, teach or suggest each element of the claim. Second, one of ordinary skill would have been motivated to combine the teachings of the cited references at the time of the invention. And third, there would have been a reasonable expectation that such a combination would have succeeded.

Applicants contend that the references cited against the rejected claims fail to support a prima facie case of obviousness.

To support a case of prima facie obviousness, Lassner et al., Blokland et al., and Palauqui et al., when combined, would have to teach or suggest all elements of the rejected claims. Moreover, there would have to have been a motive to combine them, and a reasonable expectation of the invention's success at the time of the invention. The references fail to do so.

As previously explained, Lassner et al. teach the possibility of combining the use of the prokaryote T7 RNA polymerase gene with regulatory elements which are functional in plants, for successful gene expression: "This system demonstrates the feasibility of T7 RNA polymerase-based approaches for the high-level expression of introduced gene's in plant cells" (Lassner et al., abstract, page 229, last sentence). Therefore, it is clear that the objective of these authors was to achieve a functional T7 RNA polymerase driven expression system in plants.

For a person of skill in the art, the conclusion that could be reached from Lassner et al. is that a nucleotide sequence regulated by the T7 promoter should be overexpressed when transfected into plant cells already expressing the T7 RNA

polymerase.

The T7 expression system should not necessarily interfere with the expression of endogenous genes driven by their own endogenous promoters. No allusion regarding this possibility was mentioned or insinuated in the Lassner et al. paper. Lassner et al. conclude by saying: "These experiments are a step in the development of a T7 RNA polymerase-mediated gene expression system for plants" (see discussion in page 233, first line in last paragraph). Based on this, it appears that the authors did not see any potential of using the T7 RNA polymerase for silencing purposes, but only using T7 RNA polymerase for expression purposes.

It may be mentioned at this point, that notwithstanding the many years that passed since the publication of Lassner et al., even today available commercial kits employing T7 RNA polymerase are intended for high level and efficient expression. For example, the Technical Bulletin 8033-1 (from Invitrogen Life Technologies) describes the T7 RNA polymerase uses as follows: "T7 RNA polymerase initiates synthesis at the T7 promoter sequence and produces an RNA transcript of the DNA. The RNA transcripts are used as hybridization probes for DNA and RNA blots and in situ studies, in ribonuclease protection assays where the transcript is hybridized with target mRNA sequences, to study post-transcriptional modifications including RNA splicing and polyadenylation, and for in vitro translation".

Other available T7 RNA polymerase kits from Stratagen (USA) are also intended to be used for producing: "probes for nucleic acid hybridizations, templates for in vitro translations, substrates for RNA processing studies and exon and intron mapping of genomic

DNA."

Furthermore, publications such as:

1) Alexander, W.A., Moss, B., Fuerest, T.R. (1992) Regulated expression of foreign genes in Vaccinia virus under the control of bacteriphage T7 RNA polymerase and the E. coli lac repressor. *J. Virol.* 66:2934-2942 (**Exhibit A**); and

2) Fuerest, T.R., Nile's, E.G., Studier, F.W., Moss, B. (1986). Eukaryotic transient expression system based on recombinant vaccinia virus that synthesizes Bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83:8122-8126 (**Exhibit B**),

are further examples reflecting the use of T7 RNA polymerase in mammals at the time of the invention. These articles teach one of skill in the art that the T7 RNA polymerase/T7 RNA promoter system is an efficient eukaryotic expression system.

Turning to Blockland, the Examiner has formerly argued that it would have been obvious and within the capabilities of one of ordinary skill in the art at the time the invention was made to use the RNA polymerase/pT7 system of Lassner et al. to silence a gene of interest in plant cells, for example the chs gene of Blockland et al.

Applicants respectfully traverse. Throughout Blockland et al., it is stated or implied that the level of transcription is not a prerequisite for inducing silencing. For example: "Surprisingly, even a promoterless chs transgene construct was found to suppress

the endogenous chs genes in three out of 15 transformants" (Summary, lines 23-26). And also: "It remains, however, unknown whether or not transcription of the transgene locus is required to induce co-suppression" (Summary, lines 26-28). Furthermore: "As mentioned before, the variation in transgene transcription levels in the various transformants was quite dramatic, but neither a high nor a low level correlated with co-suppression" (Results, page 866 right column, second paragraph, line 9) and: "The results described in the previous section indicate that suppression of-endogenous genes does not require highly expressed transgenes" (Results page 866, Chs suppression by a promoterless chsA transgene paragraph, first line).

Therefore, although Blockland et al. might teach crossing plants carrying different constructs in order to achieve silencing, it would not have been reasonable to introduce a "foreign potent expression system", as the T7 RNA polymerase/T7 promoter to trigger silencing, if gene silencing may be effected even with a promoterless construct.

There is nothing in Blockland et al. which would have motivated one skilled in the art to employ the expression system of Lassner et al. One of skill in the art would not use an efficient RNA polymerase, whose normal function is to transcribe mRNA from DNA sequences, with the final aim of gene expression, for the purpose of silencing such gene expression.

At the time of the invention, this concept seemed contradictory and it was definitively not obvious. The scientific rationale supporting this expression-silencing idea is in fact revolutionary.

With regards to Palauqui et al., this reference teaches co-suppression of plant host genes and transgenes that can be transmitted efficiently from silenced stocks to non-silenced scions expressing the corresponding transgene.

Given this information, it is clear that the T7-driven silencing system of the subject invention is not based on Lassner et al. in combination with Blokland et al. and/or Palauqui et al., but is a new system mediated by a different mechanism.

Accordingly, the Examiner has failed to establish the prima facie obviousness of claims 27, 28, 33, 37, and 40 as amended over these references.

Finally, on page 8 of the pending Office Action, lines 17 to 19, the Examiner states: "Further, the information in the declaration regarding the inability of the silencing signal to transduce across grafts (also discussed in item 27-30) directly contradicts the instant specification".

Applicants respectfully disagree with the characterization of the relationship between the declaration and the specification. In science, one uses samples to draw inferences about populations but a sample does not necessarily represent the population from which it is taken. By way of explanation, errors in scientific studies generally fall into one of two major categories: (a) random errors and (b) systematic errors.

A random error occurs when one takes a sample of a variable population, and by chance the sample does not perfectly represent the real population. This is always present to some degree, because populations are naturally variable. Generally, random

errors are unpredictable (make estimates either too large or too small) and have larger effects if the sample size is small, so in order to decrease the impact of random error, it is recommended to increase the sample size.

A systematic error occurs when something about the way one samples results in estimates that are consistently incorrect in some direction (either consistently too large or consistently too small). The systematic error might create bias (directional tendency towards some incorrect conclusion) and since it is caused by the sampling method, increasing sample size does not help get rid of it.

Usually, if the sample size is at least 30, one is very likely to have adequate statistical power to detect any trends that are present. Most of the times, a sample of 20 will give adequate statistical power. Statistical power is increased by decreasing the random error and we do that by increasing the sample size.

In the T7-driven silencing system of the invention, the silencing effect is restricted to the cells carrying the constructs themselves [as previously detailed in the inventor's Declaration Exhibit 4 (Meir et al. manuscript, Table 1)] and there is no transmission of the signal to other tissues as seen in other plants (as shown in Palauqui et al.) or other species (e.g. C. elegance). Therefore, the results presented in Example 4 (sample size = 6), are likely due to a statistical random error originated by the small number of the analyzed plants in the experiment (small size sampling group).

Any apparent discordance between the conclusions reached in the Example 4 of the specification which support the idea that

"silencing is transmitted from silenced stocks to non-silenced scions expressing the corresponding transgene", and the later understanding (see declaration Exhibit 4) which lead to the opposite conclusion, can be explained by a statistical error, caused by mis-chosen group size in the first experiment.

In view of the above remarks, applicants maintain that claims 27, 28, 33, 37, and 40 as amended satisfy the requirements of 35 U.S.C. §103(a). Accordingly, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 27, 28, 33, 37, 40, and 44-52.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone him at the number provided below.

No fee, other than the $510.00 fee for a three-month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully submitted,

I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Mail Stop AF, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

5/19/05

John P. White
Reg. No. 28,678

Date

John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
Tel. No. (212) 278-0400

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Regulated Expression of Foreign Genes in Vaccinia Virus under the Control of Bacteriophage T7 RNA Polymerase and the *Escherichia coli lac* Repressor

WILLIAM A. ALEXANDER,[1] BERNARD MOSS,[2] AND THOMAS R. FUERST[1]*

Department of Molecular Genetics, MedImmune Inc., Gaithersburg, Maryland 20878,[1] and Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892[2]

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The gene encoding bacteriophage T7 RNA polymerase (*T7gene1*) was placed under the control of regulatory elements from the *Escherichia coli lac* operon to construct an inducible vaccinia virus expression system consisting entirely of prokaryotic transcriptional machinery. Regulated expression of T7 RNA polymerase was necessary to construct a stable recombinant vaccinia virus harboring a T7 promoter; otherwise, uncontrolled expression led to interference with endogenous virus replication. To this end, the gene encoding the repressor protein of the *lac* operon was fused to a viral early/late promoter so that it was expressed constitutively, and the *lac* operator was interposed between a viral major late promoter and *T7gene1*. Greater than 99% repression of T7 RNA polymerase, which was relieved approximately 80-fold in the presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), was obtained. An expression cassette containing a T7 promoter-controlled β-galactosidase reporter gene was recombined into a different region of the viral genome containing *T7gene1*. A stable, double recombinant virus was isolated and grown to a high titer. In the absence of inducer, β-galactosidase expression was substantially repressed. Addition of increasing amounts of IPTG induced expression of β-galactosidase to the point of suppression of viral replication. This hybrid vaccinia virus system (Vac/Op/T7) has potential applications for the efficient bioproduction of a wide variety of gene products.

Transcriptional and regulatory elements from viral or eukaryotic sources have been used extensively for the production and characterization of recombinant proteins in mammalian cells (16). These eukaryotic expression vectors typically carry genetic elements which confer drug resistance, the ability to replicate autonomously, and regulatory control to the target gene of interest. Although use of mammalian cells is essential in many instances for the synthesis of biologically active eukaryotic proteins, stable transformants are oftentimes difficult to construct and target proteins may be expressed at relatively low levels in comparison with their bacterial counterparts. Moreover, if tight regulation of transcription is required for the expression of specific protein sequences, regulated eukaryotic transcription systems such as the metallothionein promoter (2, 21) or the mouse mammary tumor virus promoter (17, 19) may not be suitable, since they are leaky under noninduced conditions and show rather modest levels of induction. On the other hand, promoters that are highly inducible, such as those responsive to glucocorticoid hormones (18), require the presence of hormone receptors in the cell, thereby restricting the range of cell types that can be used. As an alternative approach, a wide host range mammalian cell expression system incorporating desirable and highly regulatable prokaryotic transcriptional elements might have important advantages. For instance, the high catalytic activity, inducibility, and promoter specificity of several prokaryotic transcription systems have been well characterized. Thus, a eukaryotic expression system incorporating favorable transcriptional components from bacteria may offer a highly specific and efficient method for the biosynthesis of mammalian cell-derived proteins.

In previous reports (12, 15), we described a chimeric system that provides useful expression of recombinant proteins. This system, referred to as the hybrid Vac/T7 system, is based on coinfection of cultured cells with two recombinant vaccinia viruses: one recombinant virus provides constitutive expression of bacteriophage T7 RNA polymerase which transcribes a T7 promoter-controlled target gene in the second virus. Although the Vac/T7 system has been widely used, inherent limitations exist. While the requirement for two viruses may be advantageous under some circumstances (e.g., expression of toxic proteins), it adds to the expense and complicates the protocol. For instance, optimal levels of expression are dependent on cells being infected with similar amounts of each virus. In addition, differential rates of virus replication may limit the ability to establish a spreading infection at a low multiplicity of infection (MOI). Our attempts to simplify this two-virus vector system by making a single recombinant vaccinia virus containing both T7 RNA polymerase and T7 promoter elements have been unsuccessful, presumably because of interference with viral transcription and/or replication. If expression of T7 RNA polymerase could be negatively regulated and induced upon command, a single-virus expression system may be feasible (24).

Here we describe the development of a vaccinia virus expression system composed primarily of prokaryotic transcriptional elements. A preliminary description of this work has been reported previously (1). The genes that encode the T7 RNA polymerase and regulatory elements from the *Escherichia coli* lactose operon were inserted into the genome of vaccinia virus. A recombinant vaccinia virus that constitutively expresses the *lac* repressor was constructed,

* Corresponding author.

and the activity of T7 RNA polymerase was regulated by placing it under the control of a hybrid promoter in which the *lac* operator was inserted just downstream of a late vaccinia virus promoter. The activity of T7 RNA polymerase was regulated over an 80-fold range by the *lac* repressor, and this effect was reversible by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). A secondary gene cassette that contains the *E. coli* β-galactosidase (β-GAL) gene (*lacZ*) under control of a T7 promoter was inserted into the viral genome by using *E. coli* guanine-hypoxanthine phosphoribosyltransferase (*gpt*) as a dominant selectable marker. A stable vaccinia virus recombinant was isolated and grown to high titer. Induction of β-GAL expression could be determined to the point at which the virus ceased to replicate. In this communication, we demonstrate the powerful utility of developing chimeric expression systems in mammalian cells for the production of recombinant proteins.

MATERIALS AND METHODS

Enzymes and chemicals. Restriction endonucleases were obtained from New England Biolabs or Bethesda Research Laboratories. Mycophenolic acid (MPA) was obtained from Calbiochem Corp.; hypoxanthine and xanthine were obtained from Sigma Chemical Co. MPA and xanthine were dissolved in 0.1 N NaOH, and hypoxanthine was dissolved in water and sterile filtered; the solutions were stored frozen as 10-mg/ml stocks.

Virus and cells. Vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection, propagated in HeLa cells, and purified as reported previously (20). HeLa cells S3 were grown in Eagle minimal essential medium (EMEM) supplemented with 5% horse serum. Human thymidine kinase-negative (TK^-) 143 cells (26) were grown in EMEM containing 10% fetal bovine serum (FBS) and 25 μg of 5-bromodeoxyuridine (BUdR) per ml. CV-1 and BSC-1 cells were grown in Dulbecco modified Eagle medium containing 10% FBS.

Vector construction. The sequence immediately upstream of the translation start site from *T7gene1* was modified by M13 oligonucleotide-directed mutagenesis (11) to remove the potential upstream Shine-Dalgarno sequence (GAGG) as described by Stahl and Zinn (26a) and to insert unique restriction enzyme sites. To accomplish this construction, *T7gene1* was excised from pAR1173 (5) with *Bam*HI as a 2.6-kbp DNA fragment and inserted into the *Bam*HI site of the double-stranded, replicative form of mp19. The resulting vector was termed RFmpT7gene1. An oligonucleotide with complementary sequences flanking the Shine-Dalgarno motif, 5′-ATCGTGTTCATTTAAGATCTGAATTCGGATCCT CTAGAGT-3′, was used to selectively remove the Shine-Dalgarno sequence while inserting *Bgl*II-*Eco*RI-*Bam*HI restriction sites (underlined). The *Bgl*II site abuts the 5′ end of TAAATG where the thymidine (boldface) corresponds to T7 nucleotide 3168 (6) and the T7 RNA polymerase translation start site is underlined. The mutagenized, single-stranded template was converted to the replicative form by standard techniques, and the phage were screened by plaque assay with the ^{32}P-labeled mutagenizing oligonucleotide as a probe. Positive plaques were identified, and the mutagenesis was confirmed by restriction enzyme and DNA sequence analyses. Replicative form DNA was prepared from the recombinant phage termed RFmpTF7gene1-4 (designated pTF7gene1 in Fig. 2). A second modification of *T7gene1* was performed by M13 oligonucleotide-directed mutagenesis to engineer an *Eco*RI site coincident with the translation start site of the polymerase. An oligonucleotide, TF7-11, 5′-ATG TAAATCGAATTCATTTAAGGATCCTCTAGAGT-3′, was synthesized to modify the native 5′ sequence of *T7gene1*, . . .TAAATGAACACG. . ., to . . .TAAATGAATTC. . ., thus inserting an *Eco*RI site immediately downstream of the translation start site. This modification resulted in a conservative change in the third codon from a threonine to a serine without any apparent change in T7 RNA polymerase activity. Replicative-form DNA was prepared from the positive phage and was termed RFmpTF7gene1-Eco. Construction of recombinant plasmids pT7lacOI and pP11T7gene1 is described in the legend to Fig. 2.

To generate the transfer vector, pVacHAgpt, a 1.8-kbp *Sal*I-*Hin*dIII DNA fragment containing the vaccinia virus (strain WR) hemagglutinin (HA) locus was inserted into the *Eco*RI-*Hin*dIII sites of pUC19, in which the *Sal*I and *Eco*RI sites were made flush with Klenow polymerase. The resulting vector was termed pTFHA. A set of complementary oligonucleotides containing a multiple cloning site with the restriction enzyme sites *Sst*I, *Sma*I, *Xho*I, *Bam*HI, *Sal*I, and *Eco*RI (TF62, 5′-GGAGCTCCCCGGGCTCGGAGGGATC CGTCGACTACTGAATTC-3′; TF63, 5′-GAATTCCCAG GTCGTCGACGGATCCCTCGGAGCCCGGGGAGCTC-3′) were annealed and inserted into the unique *Nru*I site in pTFHA. The multiple cloning site bisected the HA gene, and the recombinant plasmid, pVacHA, was isolated and purified. A 2.1-kbp *Eco*RI-*Sal*I DNA fragment containing the bacterial *gpt* gene encoding guanine-hypoxanthine phosphoribosyltransferase (25) regulated by the vaccinia virus P7.5 promoter was excised from pTK61-gptΔ*Bam*HI (9) and ligated to *Eco*RI-*Sal*I-cleaved pVacHA to create the vaccinia virus transfer vector, termed pVacHAgpt.

Recombinant virus isolation. Recombinant viruses vT7lacOI and vP11T7gene1 were prepared as described previously (20) by homologous recombination into the TK locus and selection for TK^- phenotypes in the presence of BUdR. To generate the recombinant virus vT7lacOIZ, CV-1 cells (3×10^6) were infected with 0.5 PFU of vT7lacOI per cell and then transfected with a calcium DNA precipitate consisting of 10 μg of supercoiled pPT7lacZ DNA, 1 μg of vT7lacOI DNA, and 14 μg of sheared calf thymus DNA. After 48 h, virus stocks were prepared by resuspending the infected cells in 1 ml of medium and freezing and thawing the mixture three times. Selection for recombinant viruses containing the bacterial *gpt* gene was accomplished by three successive rounds of plaque formation on BSC-1 cells in the presence of EMEM containing 2.5% FBS, 25 μg of MPA per ml (Calbiochem), 250 μg of xanthine per ml, and 15 μg of hypoxanthine per ml (9). The gpt^+ viruses obtained were then plated on BSC-1 cells without selection, and DNA from isolated viral plaques were analyzed by polymerase chain reaction and agarose gel electrophoresis for the inserted sequence.

β-GAL assay. Infected or transfected BSC-1 cells (10^6) were grown in 2 ml of EMEM (without phenol red) containing 2.5% FBS. The infected cells were harvested, 100 μl of $CHCl_3$ and 10 μl of 10% (wt/vol) sodium dodecyl sulfate were added, the cells were dispersed by vortexing, and the mixture was centrifuged to remove cellular debris. The supernatant was assayed for β-GAL activity by using *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (23). The reaction was performed in a 96-well microtiter plate, and the yellow color was quantitated by measuring A_{405} with a kinetic microplate reader (Molecular Devices).

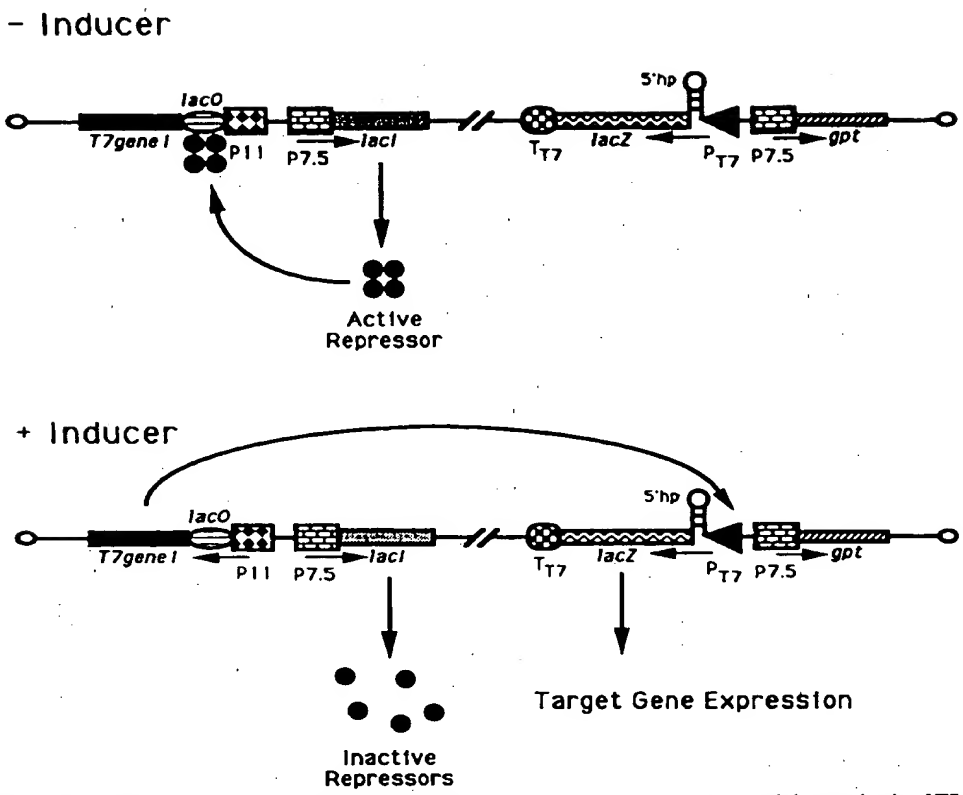

FIG. 1. Schematic representation of the interaction of *lac* repressor, inducer, and operator to control the synthesis of T7 RNA polymerase and subsequent induction of the target gene (*lacZ*). The *lac* repressor was synthesized at early and late times after infection by using the vaccinia P7.5 promoter. The active repressor binds to the *lac* operator (*lacO*) positioned between the vaccinia late promoter (P11) and the gene encoding T7 RNA polymerase (*T7gene1*). In the presence of inducer, the repressor is inactivated, and expression of T7 RNA polymerase is induced. T7 RNA polymerase initiates transcription from a T7 promoter (P_{T7}) and stops at the T7 terminator (T_{T7}). Chimeric T7-initiated transcripts are synthesized at high levels. A 5′ stem-loop sequence (5′hp) stabilizes the transcripts from degradation. The dominant selectable marker, *gpt*, allows positive selection and retention of the target gene sequences inserted into the HA locus.

RESULTS

Construction of hybrid vaccinia virus promoters to control the expression of T7 RNA polymerase. A strategy was adopted to construct a recombinant vaccinia virus that was capable of regulated expression of T7 RNA polymerase and that contained a T7 promoter-controlled target gene (Fig. 1). We felt that regulation of T7 RNA polymerase expression was necessary, as previous attempts to construct a recombinant vaccinia virus containing both T7 RNA polymerase and a T7 promoter-controlled target gene were unsuccessful apparently because of the low viability of the double recombinant (12). Since small amounts of T7 RNA polymerase can direct most of the resources of an *E. coli* cell toward expression of a specific target gene, we reasoned that a similar event was occurring, thereby impeding the ability of the recombinant virus to replicate. Therefore, we considered the use of components from the *E. coli* lactose operon coding for the *lac* repressor (*lacI*) and its cognate operator sequence (*lacO*) to regulate the T7 RNA polymerase. Our objective was to construct a recombinant vaccinia virus that constitutively expressed *lacI* and that contained an appropriately placed *lac* operator sequence strategically positioned between a vaccinia virus late promoter (P11) and the coding sequence for T7 RNA polymerase (*T7gene1*). If stringent repression of T7 RNA polymerase was achieved, then stable insertion of a T7 promoter-controlled target gene could be maintained in a second location in the viral genome. Induction of T7 RNA polymerase expression would then result in significant T7 promoter-specific transcription initiation and target gene expression.

To regulate the expression of *T7gene1*, we first modified an expression vector that contained a *lacI* gene under control of the vaccinia virus early/late P7.5 promoter to allow the insertion of secondary gene cassettes (placIm, Fig. 2A). We chose the P7.5 promoter because it is transcriptionally active at early and late times after infection (29), thereby permitting constitutive *lac* repressor expression throughout the course of infection. Earlier studies demonstrated the utility of this approach in that a recombinant virus, vlacI, containing *lacI* stably integrated into the TK locus under the control of the P7.5 promoter, was used to successfully synthesize the functionally active repressor in infected cells. The amount of repressor expressed inhibited the expression of a vaccinia virus promoter-*lac* operator-*lacZ* gene fusion by up to 99.9% (13).

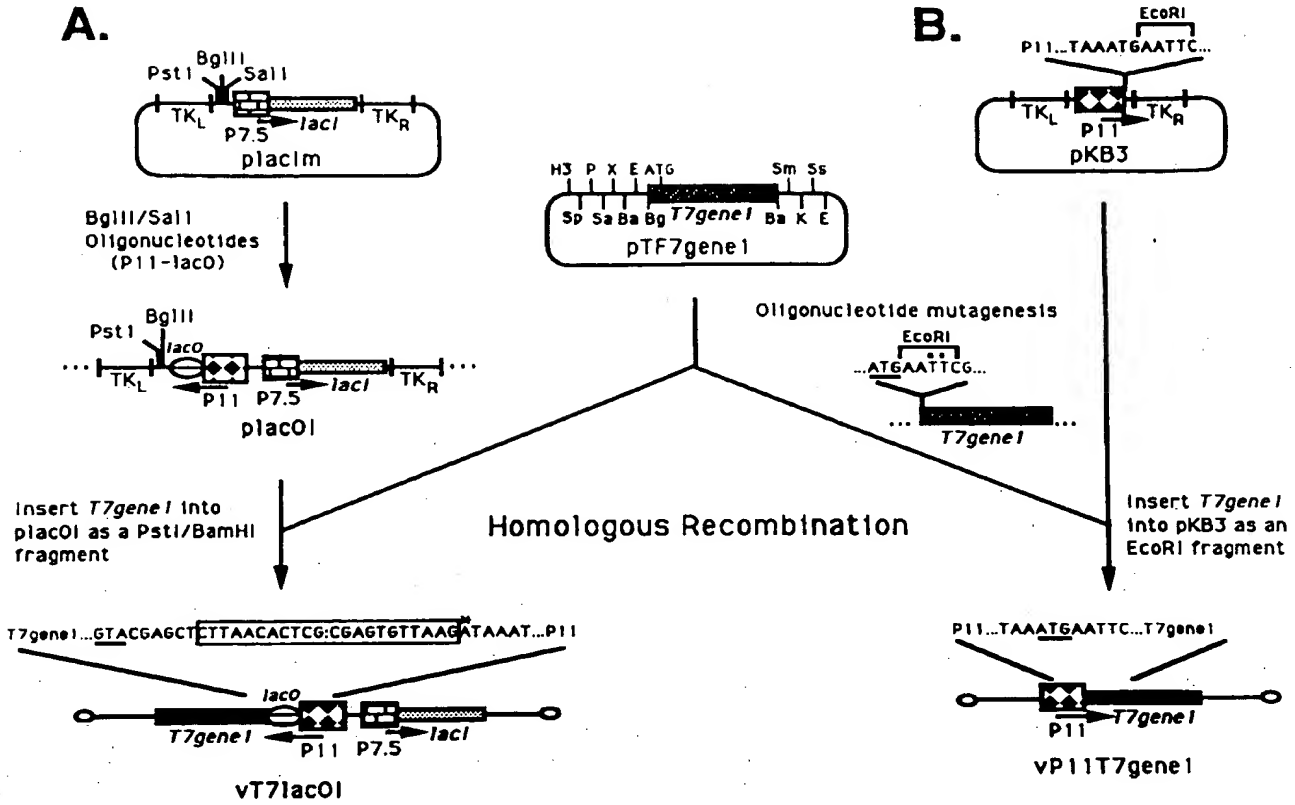

FIG. 2. Construction of recombinant vaccinia viruses which regulate the synthesis of T7 RNA polymerase. (A) Plasmid placI (13) was modified by inserting an 18-bp linker containing the restriction enzyme sites *Bgl*II, *Xho*I, and *Pst*I into the *Pst*I site, creating placIm. A 73-bp *Bgl*II-*Sal*I compatible fragment, containing the vaccinia virus late promoter (P11) and *lacO* regulatory elements (P11-lacO), composed of the complementary and overlapping oligonucleotides BA1, BA5, BA6, and TF64, was inserted into the *Bgl*II and *Sal*I sites of placIm to create placOI. The sequences of these oligonucleotides were 5'-TAGCATAGAAAAAAACAAAATGAAATTCTACTATATTTTTA-3' for BA1, 5'-CTATGCTATAAATAGAATTGTGAGCGCTCACAATTA-3' for BA5, 5'-GATCTAATTGTGAGCGCTCACAATTCTATTTA-3' for BA6, and 5'-TCGATAAAAATATAGTAGAATTTCATTTTGTTTTTTT-3' for TF64. Plasmid pT7lacOI was constructed by cleaving placOI with *Pst*I and *Bgl*II and inserting a 2.7-kbp *Pst*I-*Bgl*II fragment containing *T7gene1* from pTF7gene1 in which the Shine-Dalgarno fragment was removed. pT7lacOI was inserted into the vaccinia virus (strain WR) by homologous recombination (20), creating the recombinant virus vT7lacOI. The asterisk denotes the exchange of adenosine for guanosine in the native TAAATG sequence. The boxed area represents the 22-bp *lacO* sequence with dyad symmetry. (B) A 2.6-kbp *T7gene1* *Eco*RI DNA fragment, in which an *Eco*RI site was engineered just downstream of the translation start site (see Materials and Methods), was inserted into the *Eco*RI site of the expression vector pKB3 (8) to form pP11T7gene1. This insertion vector was recombined into the TK locus of vaccinia virus (strain WR) by standard techniques, creating the recombinant virus vP11T7gene1. The underlined translation start site represents its native location in the context of the 11-kDa protein.

We then wished to place a synthetic *lacO* adjacent to a late promoter so that repressor binding would block transcription yet not severely disturb transcription in the absence of the repressor. The optimal site for placement of the synthetic *lacO* sequence, relative to a vaccinia virus late promoter, was previously described (13). Positioning of *lacO*, a 22-bp palindrome, immediately downstream of the highly conserved TAAAT motif of late promoters satisfied these criteria. The vaccinia virus late promoter for the gene encoding the 11-kDa structural protein (P11) was used in these studies, and the hybrid promoter was referred to as P11lacO. A set of four overlapping and complementary oligonucleotides encompassing P11lacO were annealed and inserted into the *Bgl*II-*Sal*I sites of placIm to create placOI. The coding sequence for *T7gene1*, excised from pTF7gene1 in which the Shine-Dalgarno motif was removed and a unique *Bgl*II site was inserted, was juxtaposed immediately downstream of P11lacO, and the resulting plasmid was termed pT7lacOI (Fig. 2A). It was necessary to remove the Shine-Dalgarno sequence immediately upstream of the translation start site of *T7gene1* to permit fusion with the P11 promoter and subsequent propagation of the recombinant plasmid in *E. coli*. Since the P11 promoter is transcriptionally active in *E. coli*, presumably, the T7 RNA polymerase expressed is toxic to the cells (14a). In this configuration, the synthetic *lac* operator sequence was placed two bases downstream of the RNA start site of *T7gene1*. In addition, a recombinant plasmid, termed pP11T7gene1, in which *T7gene1* was fused to the naturally occurring translation start site of P11 which overlaps the TAAAT(G) sequence motif (Fig. 2B), was constructed. Although the presence of a guanosine immediately following TAAAT is not essential for late transcription,

substitution of adenosine has been shown to lower expression by approximately 25% (13). Therefore, both plasmids, pT7lacOI and pP11T7gene1, were constructed to compare the relative level of T7 RNA polymerase expressed from the natural and modified P11 promoters.

Regulation of T7 RNA polymerase expression. To determine whether genetic elements carried by pT7lacOI and pP11T7gene1 were functionally active, the recombinant plasmids were transfected separately into wild-type vaccinia virus-infected cells, and T7 RNA polymerase was assayed. In these transient assays, T7 RNA polymerase expression from pP11T7gene1 was unaffected in the absence or presence of the inducer, IPTG. However, substantial repression and subsequent induction in the presence of IPTG occurred in cells transfected with pT7lacOI (data not shown). Since T7 RNA polymerase appeared to be regulated, we proceeded to construct recombinant viruses containing these gene cassettes inserted into the TK gene by homologous recombination. TK^- recombinant viruses, designated vT7lacOI and vP11T7gene1 (Fig. 2), were purified, and the correct insertion of the gene cassettes was confirmed by Southern blot hybridization.

The ability of the recombinant virus vT7lacOI to synthesize the functionally active *lac* repressor capable of binding to its cognate operator sequence in vitro was determined by mobility shift assay. A radioactively labeled synthetic operator containing the sequence GAATTGTGAGCGCTCAC AATTC and its complement were prepared as described previously (13). The 41-bp probe was incubated with dilutions of extracts made from cells infected with vlacI, vT7lac OI, or wild-type virus. Extracts prepared from both the *lacI*-containing viruses retarded the mobility of the probe (Fig. 3). The protein-DNA complex comigrated with the complex formed from the association of the authentic repressor binding to the probe. Since known amounts of purified repressor were used as a standard, we calculated by densitometry that approximately 2×10^7 repressor tetramers per cell are present after a 24-h infection. This corresponds to approximately 1,000 active tetramers for each replicated vaccinia virus genome, in agreement with previously reported values (13).

To determine the magnitude of repression of T7 RNA polymerase, and subsequent induction of expression, cells were infected with either vT7lacO1 or vP11T7gene1 in the presence or absence of IPTG. All comparisons were made relative to the amount of T7 RNA polymerase expressed from cells infected with a TK^- recombinant virus, vP11T7 gene1, containing the unmodified P11 promoter fused to *T7gene1*. Cells lysates were prepared and tested in vitro for T7 RNA polymerase activity. In the absence of IPTG, T7 RNA polymerase was repressed by >98% at an MOI of 0.1 and by more than 99% at higher MOIs (Table 1). With the addition of IPTG, the level of T7 RNA polymerase activity was 58% of maximum levels obtained in the absence of the repressor, resulting in more than 80-fold induction. By contrast, IPTG had negligible effect on T7 RNA polymerase expression in cells infected with vP11T7gene1.

Construction of a single recombinant vaccinia virus containing *T7gene1* and T7 promoter elements. To test the feasibility of constructing an inducible, single-virus system, we sought to incorporate a T7 promoter-controlled target gene into the recombinant virus, vT7lacOI. A new transfer vector for insertion into the HA locus was constructed, as the *T7gene1* and *lacI* elements were already inserted into the TK locus. Previous studies have shown that foreign gene insertion into the vaccinia virus HA locus does not interfere with the ability of the HA recombinant virus to replicate in vitro (10). To overcome a potential problem of selection and retention of target gene sequences in the HA locus, the *E. coli gpt* gene under control of the vaccinia P7.5 promoter was used as a dominant selectable marker (9). Since mammalian cells and vaccinia virus cannot use xanthine for GMP synthesis when de novo purine synthesis is blocked, i.e., in the presence of MPA, vaccinia virus expression of *gpt* can overcome this block and allow replication to continue. The *E. coli lacZ* gene encoding β-GAL was chosen as a reporter because the assay is quantitative and there is no detectable background of β-GAL in mammalian cells (15). Moreover, we anticipated that induction of blue plaque formation, in the presence of inducer and the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), may also be used for screening. Therefore, an insertion vector was constructed, termed pPT7lacZ, containing these genetic elements with *lacZ* fused to a T7 promoter (Fig. 4). Cells infected with vT7lacOI (Fig. 2A) were transfected with pPT7lacZ, and 2 days later, cell lysates were prepared and plaqued on BSC-1 monolayers in *gpt* selection medium. A stable recombinant virus, designated vT7lacOIZ, was isolated, plaque purified three times, and grown to a high titer.

To test the inducibility of this system, BSC-1 cell monolayers were infected with 50 PFU of vT7lacOIZ with or without IPTG, and plaques were stained for β-GAL activity in the presence of X-Gal. As shown at the bottom of Fig. 4, in the absence of IPTG, plaques displayed a faint staining

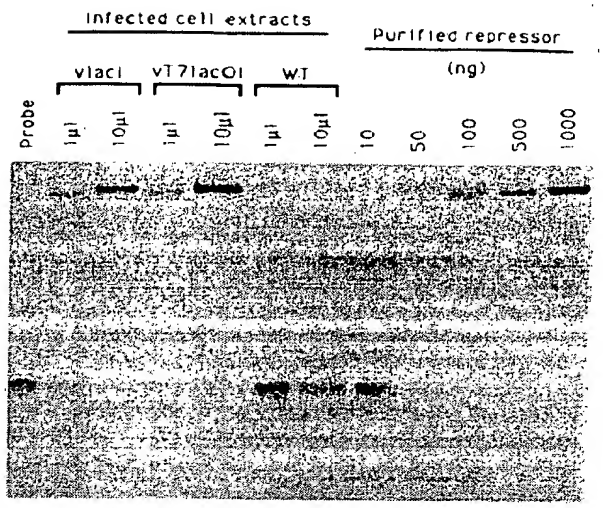

FIG. 3. Mobility shift gel of *lac* repressor binding to *lacO*. Approximately 2×10^7 CV-1 cells were infected with vlacI, vT7lacOI, or wild-type vaccinia virus at an MOI of 10. Cells were harvested 24 h after infection, and cytoplasmic fractions were prepared as described previously (13). Extracts (1 and 10 μl) were mixed with ^{32}P-labeled, double-stranded oligonucleotide probe and 50 mM poly(dI-dC). The probe was composed of the oligonucleotides PE7 and PE8 annealed together, and the nonoverlapping nucleotides were filled in with [α-^{32}P]dCTP. The sequences of these oligonucleotides were 5'-CTATGCTAGAATTGTGAGCGCTCAC AATTCTAAATAC-3' for PE7 and 5'-TCGAGTATTTAGAATTG TGAGCGCTCACAATTC-3' for PE8. In addition, various amounts of purified *E. coli lac* repressor protein were mixed with probe and poly(dIdC) to serve as a positive control. The samples were separated by 8% polyacrylamide gel electrophoresis and exposed to X-ray film. Arrowhead, *lac* repressor bound to *lacO* probe.

TABLE 1. Regulation of T7 RNA polymerase expression from vT7lacOI-infected cells[a]

Virus	Promoter junction sequence	MOI	cpm of ^{32}P incorporated (% expression)[b]		Induction (fold)
			Without IPTG	With IPTG	
vT7lacOI	P11-TAAATA-Op-T7gene1	10	1,371 (0.72)	111,811 (58.7)	81.6
		1	1,600 (0.84)	88,192 (46.3)	55.1
		0.1	2,671 (1.35)	44,572 (23.4)	16.7
vP11T7gene1	P11-TAAATG-T7gene1	10	190,480 (100)	189,527 (99.5)	None

[a] BSC-1 cell monolayers were infected with vT7lacOI or vP11T7gene1, with or without IPTG, at the indicated MOI. Cell lysates were prepared 24 h after infection. T7 RNA polymerase activity in cell lysates was assayed by using a DNA template containing a T7 promoter as described previously (15).
[b] Expression values are given as the amount (cpm) of ^{32}P-labeled ribonucleotide incorporated in a standard reaction as well as the amount (%) relative to the maximum activity obtained.

pattern, presumably because of the very low, yet detectable, level of T7 RNA polymerase expressed (see Table 1). However, plaques formed in the presence of 10 μM IPTG stained dark blue when IPTG was added at the time of infection, demonstrating the inducibility of this system.

Effect of IPTG on virus replication and β-GAL expression. Initial experiments (1) indicated that high concentrations of IPTG specifically prevented plaque formation by vT7lacOIZ. These observations led us to investigate the effect of IPTG on the growth of vT7lacOIZ. Single-step growth curves were established for the recombinant virus vT7lacOIZ and its parent lacking the PT7lacZ cassette, vT7lacOI. The addition of 1 mM IPTG at the time of infection had no effect on the ability of vT7lacOI to replicate over a 24-h period (data not shown), whereas it completely abrogated replication of vT7lacOIZ (Fig. 5A), presumably because of interference of endogenous viral transcription and/or replication. In fact, replication of vT7lacOIZ was arrested when 1 mM IPTG was added at early or late times after infection, indicating a pleiotropic adverse effect on replication. We then determined the concentration of IPTG at which virus replication became compromised (Fig. 5B). As little as 25 μM IPTG began to inhibit virus replication, and 50 μM IPTG completely abolished formation of infectious virus.

To determine the concentration of IPTG that would result in minimum inhibition of virus replication yet yield the highest level of β-GAL expression, several doses of IPTG were tested for induction. β-GAL activity present in lysates of BSC-1 cells infected 24 h earlier with 10 PFU of vT7lacOIZ per cell by using a range of 1 to 100 μM IPTG was measured quantitatively as described in Materials and Methods. A maximum level of expression was obtained by using 15 μM IPTG, which was found to have a small inhibitory effect on vT7lacOIZ replication. At this concentration of IPTG, a 5- to 10-fold induction of β-GAL activity was observed. These results demonstrate that a high degree of T7 RNA polymerase repression was achieved. This repression could be reversed, resulting in a considerable induction of β-GAL activity at low IPTG concentrations.

We next wished to determine whether maximum β-GAL expression from cells infected with vT7lacOIZ could be achieved through continuous induction at the time of infection or a burst of induction at the late times after infection. These studies, in which the single inducible virus is referred to as the Vac/Op/T7 system, were performed in comparison to the previously described hybrid Vac/T7 coinfection system (12). By using optimal conditions for each system, cells were infected with vT7lacOIZ (Vac/Op/T7) or coinfected with recombinant vaccinia viruses vTF7-3 and vTF7LZ-1 (Vac/T7) in the absence or presence of either 15 μM or 1 mM IPTG added early (2 h) or late (12 h) after infection. (Recombinant virus vTF7-3 expresses T7 RNA polymerase and vTF7LZ-1 contains the PT7lacZ cassette.) Cell extracts were prepared 24 h after infection and assayed for β-GAL expression by colorimetric assay. As shown in Table 2, the highest β-GAL activity was obtained when cells were infected at an MOI of 10 in the presence of 15 μM IPTG added at 2 h postinfection (100%). This activity is approximately twofold greater than that obtained for the coinfection system. Similar results were obtained when cells were infected with vT7lacOIZ or coinfected by using the Vac/T7 system in the presence or absence of IPTG and β-GAL protein was measured by immunoblot analysis (1). On the basis of this analysis, with purified β-GAL as a standard, we estimated that approximately 5 μg of β-GAL per 10^6 cells was made 24 h after infection by the Vac/Op/T7 system.

DISCUSSION

We have described a unique mammalian cell expression system in which prokaryotic transcriptional and regulatory elements were used to control the expression of a foreign gene carried by an animal virus host vector. We selected bacteriophage T7 RNA polymerase for its highly specific and catalytic properties and regulatory elements from the *E. coli* lactose operon for their ability to block transcription over several orders of magnitude. Vaccinia virus was chosen as a desirable vector system because it has a cytoplasmic mode of replication and it encodes mRNA modification enzymes which appear necessary for the translation of T7-specific transcripts (3, 7). In addition, vaccinia virus can replicate in a wide host range of cell types and amplifies its genome from 10,000 to 20,000 copies per cell, thereby increasing the copy number of the target gene template.

Initial attempts were made to incorporate a T7 promoter into the genome of a vaccinia virus recombinant that constitutively expressed T7 RNA polymerase. These attempts were unsuccessful, presumably because of the high catalytic properties of the polymerase, resulting in the interference with viral transcription and/or replication. Similar observations have been reported for *E. coli* with which even basal T7 RNA polymerase expression present in an uninduced cell can prevent, in some cases, target genes from being established in the same cell. In fact, the use of T7 lysozyme to inhibit such basal transcription was necessary to overcome this problem (4). These observations support the finding that relatively small amounts of T7 RNA polymerase can direct most of the resources of an *E. coli* cell toward expression of a target gene (27, 28). Furthermore, cells infected with a vaccinia virus recombinant expressing T7 RNA polymerase can direct 30% of total cellular RNA to be initiated from a T7 promoter (14). Therefore, transfer of the *E. coli lac* operator-

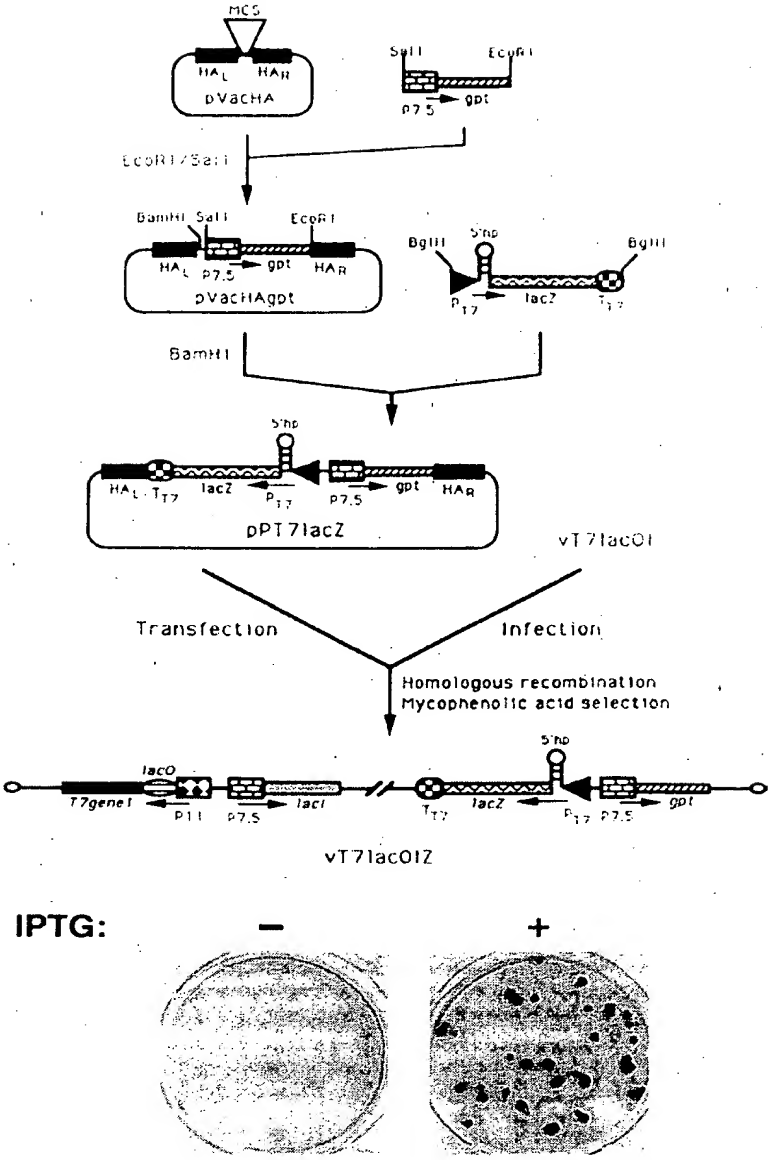

FIG. 4. Construction of a single, inducible recombinant virus. A 3.3-kbp DNA fragment containing the *E. coli lacZ* gene flanked by T7 promoter (P_{T7}) and transcription terminator (T_{T7}) elements was cleaved with *Bgl*II from pTF7LZ-1 (12) and inserted into the *Bam*HI site of pVacHAgpt. The resulting insertion vector, pPT7lacZ, was then recombined into the HA locus of recombinant vaccinia virus vT7lacOI by homologous recombination. By using MPA selection (see Materials and Methods), a recombinant virus termed vT7lacOIZ was isolated and grown to a high titer. Induction of β-GAL expression was detected by plaque assay. Confluent BSC-1 cell monolayers were infected with 50 PFU of vT7lacOIZ per well in the absence (−) or presence (+) of 10 μM IPTG added at the time of infection. After a 2-day incubation, the cell monolayers were stained with X-Gal for 8 h.

repressor system to regulate the expression of T7 RNA polymerase was essential for stable incorporation of a T7 promoter-controlled reporter gene.

The stringency of repression of T7 RNA polymerase was tested by using a hybrid promoter in which *lacO* was positioned immediately downstream of vaccinia virus late promoter P11. The optimal positioning of the *lacO* sequences was previously determined by using β-GAL as a reporter gene. Using the same promoter-operator configuration, we found that up to 99.9% repression of β-GAL expression was obtained (13). Similarly, in this study, T7 RNA polymerase was repressed greater than 99% at higher MOIs. Addition of IPTG, however, induced expression to values at least 50% of maximum, resulting in an overall

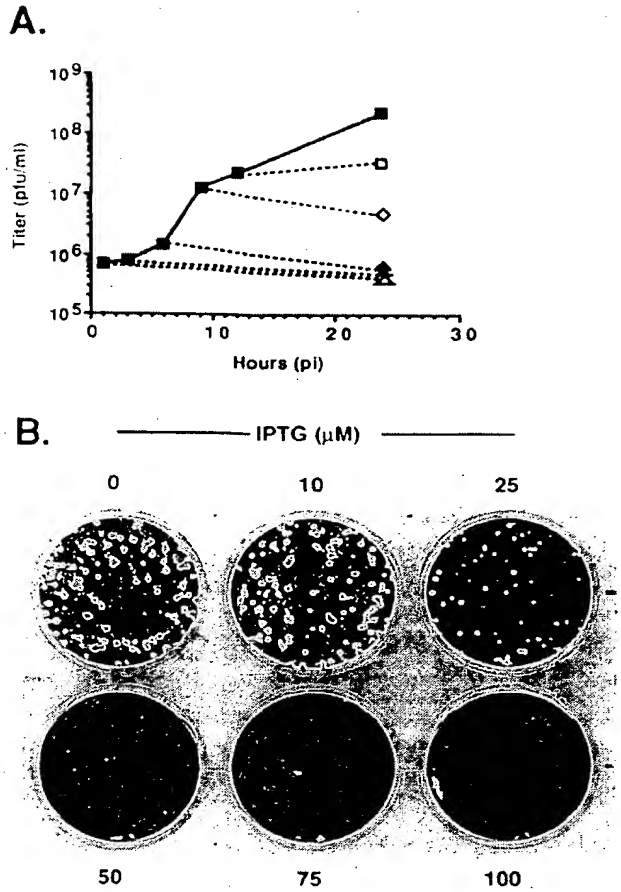

FIG. 5. Effects of IPTG induction on vT7lacOIZ replication. (A) BSC-1 cell monolayers were infected with vT7lacOIZ at an MOI of 1 PFU per cell. After 1 h, the cells were washed with EMEM containing 2.5% FBS and then overlaid with the same medium. vT7lacOIZ-infected cells (■) were incubated in the absence or presence of 1 mM IPTG added at 1 (△), 3 (+), 6 (◆), 9 (◇), or 12 (□) h postinfection (pi). Cell lysates were prepared at the indicated time points, including 24 h after infection, and titers for virus were determined by plaque assay. (B) Confluent BSC-1 cell monolayers were infected with 100 PFU of vT7lacOIZ per well in the absence or presence of the indicated concentrations of IPTG. After a 2-day incubation, the cell monolayers were stained with a solution of 0.1% (wt/vol) crystal violet in 20% ethanol.

80-fold induction in polymerase activity. Interestingly, when the reporter gene was placed under the control of a T7 promoter and tested in this system, only a sixfold induction was observed at the higher MOI (Table 2, MOI = 10). This significant basal level of uninduced β-GAL activity probably results from the barely detectable amount of T7 RNA polymerase activity formed in the absence of inducer. If necessary, a still higher degree of repression may be obtained by increasing the amount of repressor expressed and/or by using multiple copies of the operator. Since there was an approximately 1,000-fold excess of active repressor tetramer molecules present for each replicated vaccinia virus genome 24 h after infection, the latter approach of using multiple operators seems reasonable. In fact, insertion of a

TABLE 2. Comparison of β-GAL expression from the Vac/Op/T7 system versus the Vac/T7 coinfection system[a]

IPTG (mM)	Time (h)	β-GAL expression (%)[b]			
		Vac/Op/T7 at MOI of:		Vac/T7 at MOI of:	
		1	10	1	10
0		8.0	16.5	24.6	40.7
0.015	2	43.0	100.0	27.8	47.6
0.015	12	39.8	74.7	NT	NT
1.0	2	17.1	52.4	26.3	42.8
1.0	12	44.2	80.6	NT	NT

[a] BSC-1 cell monolayers were infected with vT7lacOIZ (Vac/Op/T7) or coinfected with vTF7-3 and vTF7LZ-1 (Vac/T7), with or without IPTG, at the indicated MOIs. IPTG was added either 2 or 12 h after infection. Cell lysates were prepared 24 h postinfection and assayed for β-GAL activity as described previously (15).

[b] β-GAL expression values (%) are relative to the maximum activity obtained. NT, not tested.

lac operator just downstream of a T7 promoter strongly represses transcription in *E. coli*, yet the usual high levels of expression are obtained after induction (4).

An interesting observation demonstrating the potency of the Vac/Op/T7 system was the ability to titrate the repression of virus replication by using increasing concentrations of IPTG. This effect could be due to the burden of RNA overproduction and/or read-through transcription into distally located viral transcription units, causing disruption of normal gene function. With regard to the latter, the T7 late terminator, TΦ, has been shown to terminate T7 RNA polymerase procession 80 to 90% of the time either in vitro or in vivo (5, 22). Although Northern (RNA) blot analysis of T7-initiated transcripts from the hybrid Vac/T7 system indicated that the T7 termination signal was effectively used, S1 nuclease analysis suggested that read-through transcription also occurred (14). Whether this amount of read-through impedes virus replication needs to be determined. Since TΦ is structurally similar to the rho-independent class of *E. coli* terminators, use of strong ribosomal terminators, such as rrnBT1, may be one approach for improving the stringency of transcription termination.

The general utility of this single virus Vac/Op/T7 system was demonstrated for the production of proteins at levels higher than those achieved by using the Vac/T7 coinfection system. Moreover, as optimal gene expression by using the Vac/T7 system is dependent on cells being coinfected with equal MOIs of two recombinant viruses, the single-virus Vac/Op/T7 system could be used at a low MOI to establish a spreading infection. Selection of viral mutants with a more persistent or prolonged infection phenotype in vitro, with reduced ability to replicate in vivo, may offer significant advantages and is under investigation. For these reasons, the Vac/Op/T7 system may be more economical, easier to use, and less subject to variation than a coinfection system for large-scale protein production.

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Biochemistry

Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase

(eukaryotic expression vector/genetic engineering/transcriptional signals/poxvirus)

THOMAS R. FUERST*, EDWARD G. NILES†, F. WILLIAM STUDIER‡, AND BERNARD MOSS*§

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; †Biochemistry Department, State University of New York, Buffalo, NY 14214; and ‡Biology Department, Brookhaven National Laboratory, Upton, NY 11973

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ABSTRACT **DNA coding for bacteriophage T7 RNA polymerase was ligated to a vaccinia virus transcriptional promoter and integrated within the vaccinia virus genome. The recombinant vaccinia virus retained infectivity and stably expressed T7 RNA polymerase in mammalian cells. Target genes were constructed by inserting DNA segments that code for β-galactosidase or chloramphenicol acetyltransferase into a plasmid with bacteriophage T7 promoter and terminator regions. When cells were infected with the recombinant vaccinia virus and transfected with plasmids containing the target genes, the latter were expressed at high levels. Chloramphenicol acetyltransferase activity was 400–600 times greater than that observed with conventional mammalian transient-expression systems regulated either by the enhancer and promoter regions of the Rous sarcoma virus long terminal repeat or by the simian virus 40 early region. The vaccinia/T7 hybrid virus forms the basis of a simple, rapid, widely applicable, and efficient mammalian expression system.**

Recombinant DNA technology has made it possible to develop molecular cloning vectors that allow expression of heterologous genes in prokaryotic and eukaryotic cells. Bacterial systems provide important advantages, such as ease of use and high expression, but impose a number of limitations for synthesis of eukaryotic proteins. In particular, correct folding, proteolytic processing, glycosylation, secretion, and subunit assembly may not occur or may occur incorrectly in bacteria. For these reasons, eukaryotic cells are preferred for expression of eukaryotic genes. We considered that a hybrid vector system that would utilize the highly efficient bacteriophage T7 RNA polymerase in a eukaryotic environment might have significant advantages. T7 RNA polymerase is a single-subunit enzyme, with high catalytic activity and strict promoter specificity (1, 2), that has already found wide application for *in vitro* synthesis of RNA and as the basis for high-level gene expression systems in *Escherichia coli* (3, 4). One potential problem with use of a prokaryotic RNA polymerase in a eukaryotic cell, however, is the requirement for mRNA to be processed, capped, methylated, and polyadenylylated. It would seem that at the very least, either the T7 RNA polymerase would have to be transported from its site of synthesis in the cytoplasm into the nucleus of the cell or else the eukaryotic RNA-modifying enzymes would have to function in the cytoplasm. The strategy explored here involves the introduction of the T7 RNA polymerase gene into a cytoplasmic virus.

Poxviruses comprise a widespread family of DNA viruses that transcribe and replicate their DNA in the cytoplasm. Vaccinia virus, the prototypal member of this family, has a large linear double-stranded DNA genome that encodes an entire transcription system including RNA polymerase, capping/methylating enzymes, and poly(A) polymerase (5). Additional advantages of vaccinia virus include its large capacity for foreign DNA (6), genome stability, and wide vertebrate host range. These characteristics have been utilized in the development of vaccinia virus as a eukaryotic expression vector (7, 8). In this communication, we describe the construction of an infectious recombinant vaccinia virus that synthesizes T7 RNA polymerase and the use of this recombinant in a simple, widely applicable, and highly efficient transient-expression system.

MATERIALS AND METHODS

Enzymes. Enzymes were supplied by the companies indicated and used in accordance with their instructions. Restriction endonucleases were from Bethesda Research Laboratories, New England BioLabs, or Boehringer Mannheim. The Klenow fragment of DNA polymerase I and T4 DNA ligase were from New England Biolabs. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

Virus and Cells. Vaccinia virus (strain WR) was originally obtained from the American Type Culture Collection, replicated in HeLa cells, and purified as reported previously (9). HeLa cells were grown in Eagle's minimal essential medium supplemented with 5% horse serum. Human TK^- 143 cells (10) were grown in Eagle's medium with 10% fetal bovine serum (FBS) and 25 μg of 5-bromodeoxyuridine (BrdUrd) per ml. CV-1 monkey kidney cells were grown in Dulbecco's modified Eagle's medium containing 10% FBS.

Plasmids. pGS53 contains the vaccinia virus *P7.5* promoter, unique *Bam*HI and *Sma* I restriction sites for insertion of foreign genes, and thymidine kinase (TK) flanking sequences. It differs from the previously described pGS20 vector (11), principally in the use of pUC13 (12) plasmid instead of pBR328 (13) and TK flanking sequences derived from the Wyeth strain of vaccinia virus instead of the WR strain.

Preparation and Cloning of DNA. Recombinant plasmids were constructed and used to transform bacteria following the methods of Maniatis *et al.* (14). Plasmids were prepared by the alkaline $NaDodSO_4$ method as described by Birnboim and Doly (15) and purified by CsCl/ethidium bromide equilibrium density gradient centrifugation. Plasmids were routinely checked by agarose gel electrophoresis to ensure that the majority of DNA was in the supercoiled configuration. DNA fragments were isolated from low-melting-point agarose gels, following the Elutip-d (Schleicher & Schuell) method. DNA was extracted from purified virus as described (9).

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Abbreviations: TK, thymidine kinase; CAT, chloramphenicol acetyltransferase; *cat*, gene encoding CAT; FBS, fetal bovine serum; BrdUrd, 5-bromodeoxyuridine; kbp, kilobase pair(s).

§To whom reprint requests should be addressed.

Isolation of Recombinant Virus. CV-1 cells were infected with 0.05 plaque-forming units of wild-type vaccinia virus per cell and transfected with calcium phosphate-precipitated plasmid as described (9). Recombinant viruses formed by homologous recombination of the foreign gene into the TK locus were selected by plaque assay on TK^- 143 cell monolayers in the presence of BrdUrd (25 μg/ml). TK^- recombinant virus plaques were distinguished from spontaneous TK^- mutant virus by DNA·DNA dot blot hybridization (9). After two consecutive plaque purifications, recombinant virus was amplified by infecting TK^- 143 cell monolayers in the presence of BrdUrd, and then large stocks were made in HeLa cells without selection.

Transient Assay Conditions. For standard assays (16), CV-1 cells were grown to 80% confluence in 25-cm^2 flasks ($\approx 2.5 \times 10^6$ cells) and infected with either purified wild-type or recombinant vaccinia virus at a multiplicity of 30 plaque-forming units per cell. The virus was allowed to adsorb for 30 min at 37°C with occasional rocking of the plate. The inoculum was then removed and 1 ml of calcium phosphate-precipitated DNA (10 μg of recombinant plasmid and 10 μg of salmon sperm DNA) was added. After 30 min at room temperature, fresh medium containing 2.5% FBS was added and the flask was incubated at 37°C. Cells were harvested at 24 hr after infection and suspended in the indicated buffer.

To compare the levels of expression obtained with the vaccinia/T7 and more conventional transient systems, care was taken to follow the conditions described by Gorman *et al.* (17). On the day prior to transfection, low passage-number (<10 passages) CV-1 cells were plated at a density of 2.5×10^6 cells per 25-cm^2 flask and were refed with fresh medium containing 10% FBS at 3 hr before transfection. A 2-min glycerol shock was performed at 3.5 hr after transfection, and cell lysates were prepared at 48 hr.

T7 RNA Polymerase Assay. Transfected or infected CV-1 cells ($\approx 2.5 \times 10^6$) were resuspended in 0.25 ml of 0.01 M Tris·HCl, pH 7.6/0.01 M NaCl/1.5 mM $MgCl_2$ and Dounce homogenized. After centrifugation, 1.5 μl of cytoplasmic supernatant was assayed for T7 RNA polymerase at 37°C in 25-μl mixtures containing 40 mM Tris·HCl (pH 8.0); 8 mM $MgCl_2$; 2 mM spermidine; 50 mM NaCl; 1 mM each ATP, CTP, and UTP; 5 μM [α-^{32}P]GTP; 30 mM dithiothreitol; 1 μg of pTFLZ-1 template; and 40 units of RNasin (Promega Biotec, Madison, WI). At various times the reactions were stopped by addition of 0.05 ml of 50 mM EDTA/0.1% $NaDodSO_4$ containing 100 μg of proteinase K per ml, followed by incubation for 60 min at 37°C. Samples were applied to DE-81 paper (Whatman) and washed thrice for 5 min in 5% Na_2HPO_4, once with water, and once with 95% ethanol. The samples were dried, and radioactivity was measured in a scintillation spectrophotometer.

β-Galactosidase Assay. Infected or transfected CV-1 cells ($\approx 2.5 \times 10^6$) were suspended in 1 ml of phosphate-buffered saline, frozen and thawed three times, and dispersed by sonication. The cellular debris was removed by centrifugation and the supernatant was assayed for β-galactosidase activity, using *o*-nitrophenyl β-D-galactopyranoside as described by Miller (18). After 30 min at 37°C, the reaction was stopped by addition of 1 M Ca_2CO_3 and the yellow color was quantitated by measuring absorbance at 420 nm. β-Galactosidase activity was recorded as nmol of *o*-nitrophenol produced in 30 min per 2.5×10^6 cells.

Chloramphenicol Acetyltransferase (CAT) Assay. Infected or transfected CV-1 cells ($\approx 2.5 \times 10^6$) were suspended in 0.2 ml of 0.25 M Tris·HCl (pH 7.5). After three freeze–thaw cycles, the lysates were dispersed by sonication, and the suspensions were assayed for enzyme activity as described by Mackett *et al.* (11).

RESULTS

Construction of a Recombinant Vaccinia Virus Containing the Bacteriophage T7 RNA Polymerase Gene. Procedures for the insertion and expression of foreign genes in vaccinia virus have been described in detail (9, 11). Vaccinia virus promoters are required to regulate transcription of the DNA which is introduced by homologous recombination into the 185-kilobase-pair (kbp) linear double-stranded DNA genome. To facilitate the use of vaccinia virus as a vector, a series of plasmids were made that contain a vaccinia virus promoter, restriction endonuclease sites for insertion of foreign DNA, and flanking vaccinia TK sequences to direct recombination into the TK locus of the genome (9, 11). For this study, we used the plasmid pGS53, which contains a promoter termed *P7.5* with early and late regulatory signals (19), to permit continuous expression of foreign genes. A 2.65-kbp DNA fragment, containing the entire T7 gene *1* coding region for T7 RNA polymerase, was excised with *Bam*HI from plasmid pAR1173 (20) and inserted into the unique *Bam*HI site of pGS53 (Fig. 1). A plasmid designated pTF7-3, with the vaccinia promoter and T7 RNA polymerase in proper orientation, was isolated from transformed *E. coli*. Plasmid pTF7-3 was used to transfect cells that were infected with vaccinia virus, and then TK^- recombinant virus plaques were selected. Correct insertion of the T7 RNA polymerase gene in the

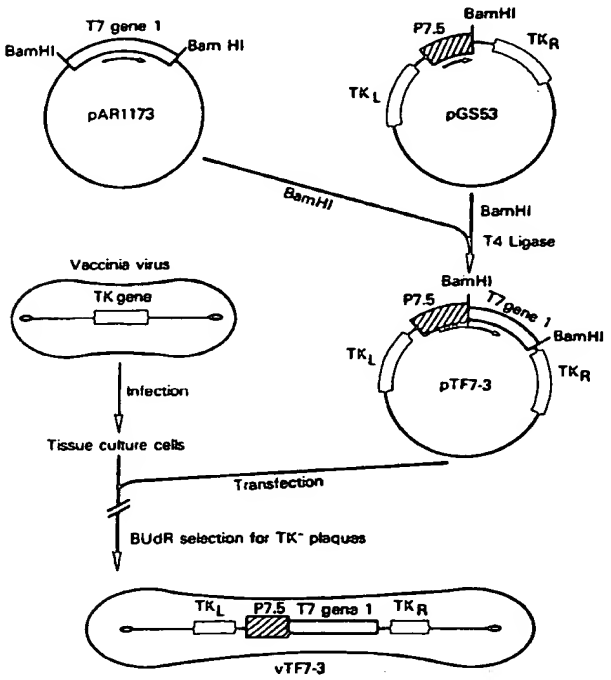

FIG. 1. Insertion of bacteriophage T7 gene *1* into the genome of vaccinia virus. A 2.65-kbp *Bam*HI fragment containing T7 gene *1* was excised from pAR1173 and inserted into the unique *Bam*HI site of pGS53 to form pTF7-3. In the latter plasmid, the coding sequence for T7 RNA polymerase is downstream of the vaccinia *P7.5* promoter and the chimeric gene is flanked by the left (TK_L) and right (TK_R) vaccinia TK gene sequences. DNA segments are not drawn to scale. CV-1 cells were infected with vaccinia virus and transfected with pTF7-3. After 48 hr, the cells were harvested and the virus was plaqued on TK^- cells in the presence of BrdUrd ("BUdR"). Virus plaques were amplified and screened by dot blot hybridization to T7 gene *1* DNA.

genome of vTF7-3 was confirmed by DNA blot hybridization. Either plasmid pTF7-3 or recombinant virus vTF7-3 was used for expression studies.

Expression of T7 RNA Polymerase in Mammalian Cells. Previous studies (16) indicated that plasmids containing genes under control of a vaccinia virus promoter are specifically transcribed in cells infected with vaccinia virus. Similarly, we wished to determine whether T7 RNA polymerase would be expressed when vaccinia virus-infected cells were transfected with the plasmid pTF7-3. T7 RNA polymerase activity in cell lysates was assayed using a DNA template containing a T7 promoter. Control experiments established that RNA polymerase activity measured with this template was not increased after vaccinia virus infection (Fig. 2). When vaccinia virus-infected cells were also transfected with pTF7-3, however, a significant increase in activity was observed (Fig. 2). This activity was not detected when a similar DNA template lacking the T7 promoter was used in the enzyme assay. Additional experiments demonstrated that T7 RNA polymerase activity was not detected when uninfected cells were transfected with pTF7-3 or when infected cells were transfected with a plasmid containing the T7 gene *1* without a vaccinia promoter (data not shown).

Next, we wished to determine whether higher levels of T7 RNA polymerase would be expressed when the T7 gene *1*, under control of a vaccinia promoter, was integrated into the vaccinia virus genome. As shown in Fig. 2, vTF7-3-infected cell extracts contained several times more T7 RNA polymerase activity than was present in cells that had been transfected with pTF7-3 in the presence of wild-type vaccinia virus. This quantitative difference between recombinant virus and transient-expression systems was consistent with previous observations (16).

Construction of Plasmids Containing Target Genes with T7 Promoters. To determine whether bacteriophage T7 RNA polymerase made under control of vaccinia virus can function in mammalian cells, we constructed plasmids containing target genes flanked by T7 promoter and termination regulatory elements. Plasmid pAR2529 (A. H. Rosenberg, J. J. Dunn, and F.W.S., unpublished work) contains the T7 gene *10* (*φ10*) promoter separated by a unique *Bam*HI site from the T7 terminator *Tφ*, which has a potential stem–loop structure followed by a run of thymidylate residues (2). As targets, we chose the *E. coli* β-galactosidase gene (*lacZ*) and the CAT gene (*cat*) derived from the Tn*9* transposon. These genes are ideal for expression systems because simple and quantitative assays are available for the enzyme products and there is no detectable background activity in mammalian cells (11, 17, 21). The *lacZ* or *cat* gene, each with an associated ATG translation initiation codon, was inserted into the unique *Bam*HI site of pAR2529 (Fig. 3). Plasmids with *lacZ* and *cat* in the correct orientation were designated pTF7LZ-1 and pTF7CAT-1, respectively.

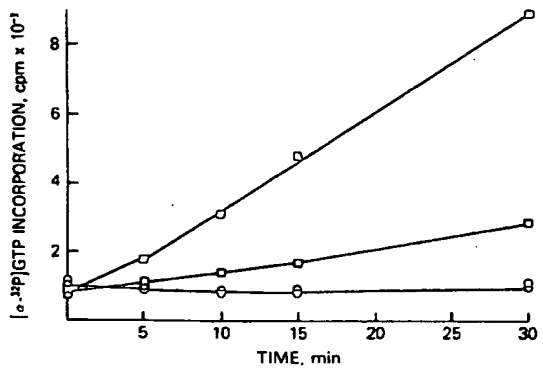

FIG. 2. Synthesis of T7 RNA polymerase. CV-1 cells were infected and transfected as described in *Materials and Methods*. After 24 hr, extracts from uninfected cells (○), cells infected with vaccinia virus (○), cells infected with vaccinia virus and transfected with pTF7-3 (□), or cells infected with vTF7-3 (□) were prepared and assayed for T7 RNA polymerase. Incorporation of [α-32P]GTP into RNA that bound to DEAE-cellulose filters was measured.

Transient Expression of β-Galactosidase. Above, we showed that cells infected with vaccinia virus and transfected with a plasmid containing the T7 gene *1* under control of a vaccinia virus promoter synthesized T7 RNA polymerase. We now asked whether vaccinia virus-infected cells would express β-galactosidase if they were transfected with plasmids containing the T7 gene *1* under control of a vaccinia promoter and the *lacZ* gene under control of a T7 promoter. Transient expression would thus depend on vaccinia virus-regulated synthesis of T7 RNA polymerase, the intracellular functioning of the T7 RNA polymerase, the production of translatable mRNA from a T7 promoter, and the synthesis of a prokaryotic enzyme. As shown in Table 1, β-galactosidase was detected in cell lysates. Omission of either vaccinia virus or the plasmid containing the T7 RNA polymerase gene prevented expression of β-galactosidase. Negative results also were obtained with either the T7 gene *1* or the *lacZ* gene was oriented oppositely with respect to the vaccinia or T7 promoter, respectively (data not shown).

In the above experiments, both the T7 gene *1* and the *lacZ* gene were transcribed from plasmids. Since more T7 RNA polymerase is made when gene *1* is integrated into vaccinia virus (Fig. 1), we anticipated that higher amounts of β-galactosidase would be produced if cells were infected with recombinant vaccinia virus vTF7-3 and then were transfected with the *lacZ* plasmid pTFLZ-1. As shown in Table 1, more than twice as much β-galactosidase was made when T7 RNA polymerase was expressed by a recombinant virus than when it was expressed from a plasmid.

We also wished to compare the transient expression of *lacZ* under control of T7 and vaccinia virus promoters. The vaccinia virus promoter used, *P7.5*, was the same as that regulating expression of T7 gene *1*. When cells were infected with vaccinia virus and transfected with the plasmid containing *lacZ* under control of the vaccinia promoter, β-galactosidase activity was about 5% of that obtained with the vaccinia/T7 transient system.

An additional comparison was made with a recombinant vaccinia virus that has the *lacZ* gene, under control of the *P7.5* promoter, inserted into the vaccinia virus genome. In this case, there was no need to transfect with a plasmid containing a target gene. Nevertheless, a mock transfection with pUC18 vector was performed in order to keep conditions equivalent. The data (Table 1) indicated that *lacZ* expression was actually about 5-fold higher with the vaccinia/T7 transient system than with the *lacZ* recombinant vaccinia virus. However, if optimal conditions for recombinant virus infection are used (i.e., transfection with calcium phosphate-precipitated DNA is not performed) then the *lacZ* recombinant expresses β-galactosidase at a level that is 2–3 times higher than with the vaccinia/T7 transient transfection system (data not shown).

Transient Expression of CAT. We considered it most important to compare the vaccinia/T7 transient expression system with more conventional ones used in mammalian cells. Since *cat* is the most common target gene used for comparison of expression levels (17, 22), experiments similar to those performed with *lacZ* were repeated (Table 1). As in the case of *lacZ*, we found that transient expression of *cat* from the T7 promoter was higher when the T7 gene *1* was integrated into vaccinia virus than when it was cotransfected on a second plasmid and that expression was much higher in

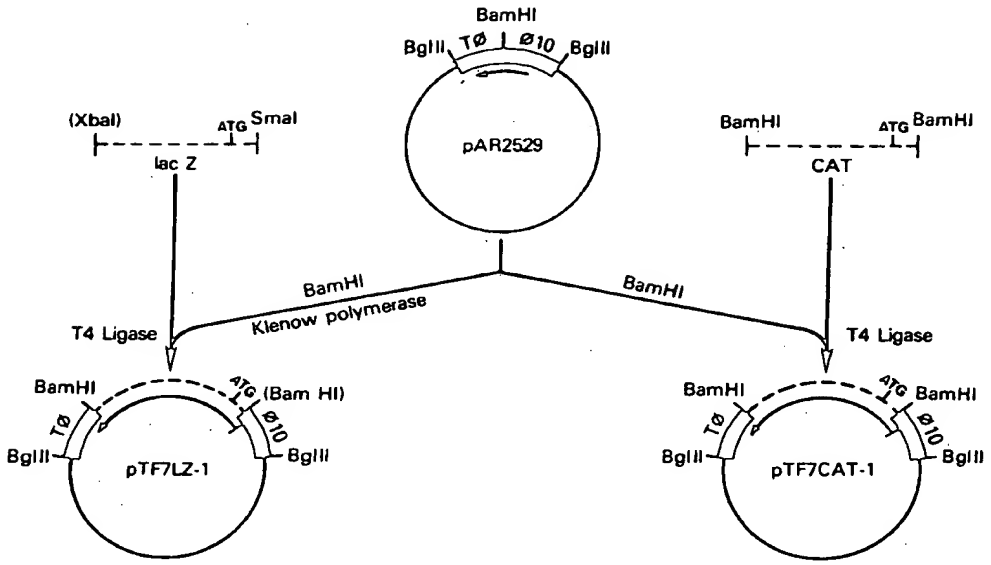

FIG. 3. Construction of plasmids containing target genes flanked by T7 promoter and terminator sequences. A 3.2-kbp DNA segment containing the *lacZ* gene with translation and termination codons was obtained by cleavage of pWS61 (provided by A. Majmdar, National Institutes of Health) with *Xba* I, filling in the staggered end with the Klenow fragment of DNA polymerase I and dNTPs, and cleaving with *Sma* I. The fragment was then blunt-end-ligated to pAR2529 that had been cleaved with *Bam*HI and treated with Klenow fragment. The resulting plasmid, pTF7LZ-1, has the β-galactosidase coding sequence flanked by the T7 *ϕ10* promoter and *Tϕ* terminator. Similarly, a 0.7-kbp *Bam*HI fragment from pGS30 (11) containing the CAT gene was ligated to *Bam*HI-cleaved pAR2529 to form pTF7CAT-1.

the vaccinia virus/T7 hybrid system than when the *cat* gene was expressed directly from the vaccinia promoter.

For further comparison, CV-1 cells were transfected with pRSVcat or pSV2cat, which contain the same *cat* DNA fragment derived from the Tn*9* transposon as pTF7CAT-1 and either the enhancer and promoter from the Rous sarcoma virus (RSV) long terminal repeat or the simian virus 40 (SV40) early region (17, 22). In order not to prejudice the results in favor of the vaccinia/T7 system, previously described experimental conditions for expression of pSV2cat and pRSVcat were employed. Thus, low-passage CV-1 cells and glycerol shock were used, and cell lysates were made at 48 hr after transfection. The extracts were diluted and tested for CAT activity. As illustrated by the autoradiogram in Fig. 4, several hundred times more CAT was made in the vaccinia/T7 system than with either pSV2cat or pRSVcat. More quantitative results, obtained by scintillation counting, indicated that 4560 nmol of chloramphenicol was acetylated per 2.5×10^6 cells using the vaccinia/T7 system, compared to

Table 1. Transient expression of β-galactosidase and CAT

Vaccinia virus	Plasmid 1		Plasmid 2		Expression	
	Promoter	Gene	Promoter	Gene	LacZ	CAT
WT	VV	T7 *1*	T7	*lacZ*	1100	
–	VV	T7 *1*	T7	*lacZ*	0	
WT			T7	*lacZ*	0	
T7 gene *1*			T7	*lacZ*	2406	
WT			VV	*lacZ*	137	
lacZ	(control plasmid)				480	
WT	VV	T7 *1*	T7	*cat*		1650
–	VV	T7 *1*	T7	*cat*		0
WT			T7	*cat*		0
T7 gene *1*			T7	*cat*		4330
WT			VV	*cat*		300
cat	(control plasmid)					1430

CV-1 cells were uninfected (–) or were infected with wild-type (WT) or recombinant (T7 gene *1*, *lacZ*, or *cat*) vaccinia virus. The recombinant viruses had the foreign gene under control of the vaccinia virus *P7.5* promoter and inserted into the TK locus. Uninfected or infected cells were transfected with one or two plasmids containing either the T7 gene *1*, *lacZ*, or the *cat* gene under control of the vaccinia virus *P7.5* promoter (VV) or the T7 *ϕ10* promoter. When cells were infected with a recombinant vaccinia virus containing either *lacZ* or *cat*, transfection was carried with a control pUC18 plasmid. Cells were harvested after 24 hr and lysates were assayed for β-galactosidase (LacZ) or CAT. Expression is given as nmol of product formed in 30 min per 2.5×10^6 cells.

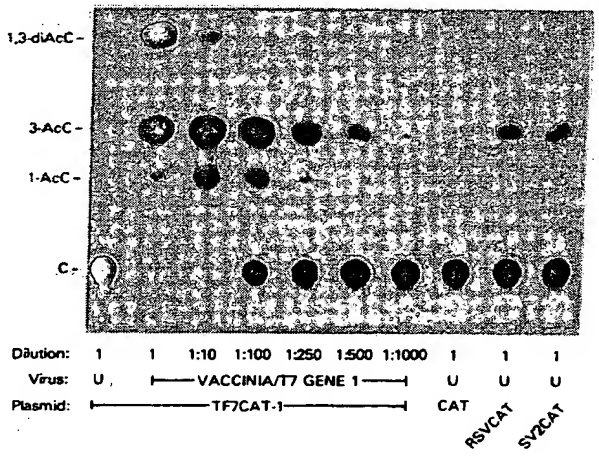

FIG. 4. Comparison of transient-expression systems. Cell lysates were prepared at 48 hr after infection with vTF7-3 (vaccinia/T7 gene *1*) and/or transfection with the indicated plasmid and assayed for CAT. (Virus "U" indicates uninfected cells.) Samples were spotted on a silica gel plate and chromatographed. An autoradiogram is shown, with the positions of chloramphenicol (C) and acetylated forms of chloramphenicol (AcC) indicated.

only 6.8 nmol with pSV2cat or 9.1 nmol with pRSVcat. This difference was even greater when glycerol shock was omitted and cells were lysed at 24 hr after transfection (data not shown).

DISCUSSION

Genetic engineering has been used extensively to express eukaryotic genes in prokaryotes and vice versa. Previously, however, this involved exchanges of coding sequences. The more radical transfer of a transcription system from a prokaryote to a eukaryote opens up new opportunities for regulating gene expression. In this study, we have integrated a functional bacteriophage RNA polymerase gene into a eukaryotic virus. Our choice of a single-subunit RNA polymerase with stringent promoter specificity and a cytoplasmic DNA virus that encodes its own RNA-modifying enzymes may have been critical to the success of the hybrid system for gene expression.

Transcription of the T7 RNA polymerase gene in vaccinia virus-infected cells is accomplished by the vaccinia RNA polymerase and is therefore dependent on the fusion of the bacteriophage gene to a vaccinia promoter. Expression of T7 RNA polymerase could be obtained either by transfecting vaccinia virus-infected cells with a plasmid containing the chimeric gene or by integrating the gene into a nonessential site within the genome of vaccinia virus. Recombinant viruses were stable, could be grown to high titer, and produced higher levels of T7 RNA polymerase than transfected plasmids.

Target genes chosen for expression by T7 RNA polymerase were inserted into a plasmid at a unique restriction site separating a T7 promoter from a T7 terminator. For these studies, the target genes (*lacZ* and *cat*) had associated translational initiation codons, but other plasmid vectors that supply the ATG and appropriate flanking nucleotides could be used for production of fusion proteins. The key step was to transfect these plasmids into cells that were infected with the vaccinia virus recombinant which expressed the T7 RNA polymerase gene. We compared the synthesis of β-galactosidase and CAT by the vaccinia/T7 hybrid system to that which occurred with a straight vaccinia transient-expression system (in which the target gene has a vaccinia promoter) and to a conventional transient-expression system (in which either the enhancer and promoter from the long terminal repeat of Rous sarcoma virus or the early region of simian virus 40 was used). The vaccinia/T7 system was 15- to 20-fold more efficient than the straight vaccinia system and 400- to 600-fold more efficient than the conventional system.

The efficiency of the vaccinia/T7 transient system, compared to that of more conventional ones, may be attributed to several factors. Since it is possible to infect tissue culture cells synchronously with vaccinia virus, all cells should have T7 RNA polymerase. Moreover, T7 RNA polymerase is a very active enzyme with a 5-fold faster elongation rate than that of *E. coli* RNA polymerase (1). Evidently the bacteriophage enzyme is able to function within the eukaryotic milieu. In addition, since the vaccinia virus RNA-modifying enzymes and, presumably, T7 RNA polymerase are localized in the cytoplasm, the transfected plasmid does not have to enter the nucleus for transcription and the mRNA produced does not have to be processed and transported back to the cytoplasm for translation. In this regard, a previous report (23) suggests that the transport of calcium phosphate-precipitated DNA into the nucleus may be a limiting factor in mammalian transient-expression systems.

Previous experience with vaccinia virus expression vectors should be directly applicable to this new system. For example, there is abundant evidence that eukaryotic proteins made in vaccinia virus-infected cells are properly processed, glycosylated, and transported to the plasma membrane (refs. 24 and 25 and refs. therein). In addition, because of the wide host range of vaccinia virus, a variety of vertebrate cells of mammalian and avian origin are suitable.

The T7 promoter is especially versatile because of its use for *in vitro* synthesis of translatable mRNA and in prokaryotic expression vectors (3, 4). Development of the vaccinia/T7 hybrid virus system makes it possible to use previous or slightly modified plasmid vectors for a third purpose: efficient expression of genes in eukaryotic cells. We have concentrated our initial efforts on developing the vaccinia/T7 system for analytical purposes because of the simplicity and potentially wide application provided by transient expression of target genes in plasmids. However, even higher levels of expression, which are more suitable for production purposes, can be achieved when both the T7 RNA polymerase gene and the target gene are carried by vaccinia virus vectors (T.R.F. and B.M., unpublished data).

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